Short Communication

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine and Cyclic GMP Stimulate Phosphorylation of Nuclear Proteins from Rat Liver*

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N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) is one of the most potent mutagens[1,2] which methylate guanosine residues in DNA.[3] It is also known that *N*-nitroso compounds stimulate the synthesis of cGMP in several tissues.[4,5] Furthermore, cGMP has been implicated in regulation of cell division.[6] This study addressed the question of whether the carcinogenic properties of MNNG arise from its double action: from (a) its above-mentioned mutagenic effect and (b) its possible effect on cell division exerted via stimulation of cGMP synthesis. We have studied the effects of MNNG on cGMP levels in the nuclei of liver cells and the activation of protein phosphorylation by MNNG in isolated nuclei. The data indicate that nuclear cGMP levels are increased as a result of incubation with MNNG, that cGMP-dependent protein kinase and its substrate proteins are present in the nuclei of hepatocytes.

Experimental

Liver nuclei were prepared from livers of normal or partially hepatectomized male Sprague-Dawley rats (160–200 g) according to the method of Blobel and Potter[7] as modified by Koide *et al.*[8] The purity of nuclear fractions was checked by demonstrating that no lactate dehydrogenase or fumarase activity could be detected. The overall preparation time for nuclei starting from liver slices or homogenates was 90 min.

Partial hepatectomy was performed under ether anaesthesia according to the method of Higgins and Anderson.[9] The left and median lobes, which constitute about 70 % of total liver mass were resected. Sham operated rats were used as control.

cGMP was extracted from nuclei and tissue slices by a modification of the method described by Folbergrová[10] and assayed by radioimmunoassay according to Steiner *et al.*[11] The nuclei were prepared from tissue slices, incubated with MNNG (100 μM) and IBMX (1 mM) using the method of Koide *et al.*[8] except that a SW-40 rotor (200,000 × *g* × 15′) was used to speed up the preparation of nuclei.

Guanylate cyclase activity was assayed by the method described previously by Bartfai *et al.*[12] The final reaction mixture (200 μl volume) contained 50 mM triethanolamine/HCl (pH 7.6), 2 mM $MnCl_2$, 1 mM GTP yielding 0.99 mM MnGTP, 1 mM MnGTP, 1 mM IBMX (3-isobutyl-1-methylxanthine) and 0.04-0.08 mg of nuclear protein. The incubation was started by addition of the protein and was carried out at 37 °C for 5 min. The cGMP formed was assayed by radioimmunoassay. The MNNG activation of nuclear guanylate cyclase was carried out in the same buffer as in the phosphorylation experiments (*i.e.*, 50 mM HEPES, pH 7.4; 10 mM $MgCl_2$ and 1 mM EGTA) containing different

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concentrations of MNNG (10^{-8}–10^{-3} M). The activation with MNNG was carried out for 15 min; thereafter, the nuclei were spun down in a bench-top centrifuge for 5 min ($1000 \times g$), 100 μg purified nuclei were added to the guanylate cyclase mixture, and the enzyme activity assayed using Mn-GTP (1 mM) as substrate.

Protein phosphorylation experiments were carried out by incubating freshly prepared nuclei or homogenate with [γ^{32}P]-ATP in an incubation mixture containing, in 200 μl final volume, HEPES (4-(2-hydroxethyl)-1-piperazine-ethanesulfonic acid) 50 mM (pH 7.4), $MgCl_2$ 10 mM, EGTA (ethyleneglycol-bis-aminoethyl ether-*N,N'*-tetraacetic acid) 1 mM, and protein 100–200 μg at 37 °C. The test agents cGMP, cAMP and MNNG were added to the appropriate test tube. Preincubation with cGMP and cAMP lasted for 1 min while with MNNG the duration of the preincubation was 15 min. The reaction was initiated by the addition of 5 μCi [γ^{32}P]-ATP. The incubation was stopped by adding 100 μl of a SDS-stop solution containing SDS (sodium dodecyl sulfate) 6 % (w/v), 2-mercaptoethanol 6 % (v/v) and glycerol 15 % (v/v) in trisphosphate buffer (25 mM, pH 6.9) and bromphenol blue (0.005 %) as a tracking dye. The samples were thereafter boiled for 3 min. These samples were kept refrigerated usually overnight and warmed in a water bath before the SDS/polyacrylamide gel electrophoresis was run on the following day.

The discontinuous polyacrylamide gel electrophoresis was carried out according to Rudolph and Krueger.[13] Autoradiography was performed on a Kodak X-Omat (XR-5) film at −70 °C for 24–72 h. The intensity of the autoradiogram was measured by using a densitometer.

Protein was determined according to Lowry *et al.*[14] with bovine serum albumin as standard.

Results and discussion

Incubation of the nuclear fraction of liver cells with MNNG for 15 min at 37 °C led to a dose-dependent stimulation of the nuclear guanylate cyclase activity (Fig. 1).

The cGMP concentration of isolated nuclei was maximal at 15 min and declined subsequently (Fig. 1, insert) indicating that MNNG can cause a transient elevation of cGMP levels.

To examine whether the elevated cGMP levels stimulate protein phosphorylation, experiments were carried out using isolated nuclei from normal and hepatectomized rats.

Stimulation of the phosphorylation of a 23,000 dalton and of a 57,000 dalton protein by MNNG (0.1 mM) (Fig. 2A) and cGMP (5 μM) (Fig. 2B) was observed in isolated nuclei when [γ^{32}P]-ATP was used as a phosphate donor. The phosphorylation of the 23,000 and 57,000 dalton proteins was specific for the experimental situation. In the control situation, the intensity of these two bands was less than 10 % of the experimental bands. Similar phosphorylation patterns were observed

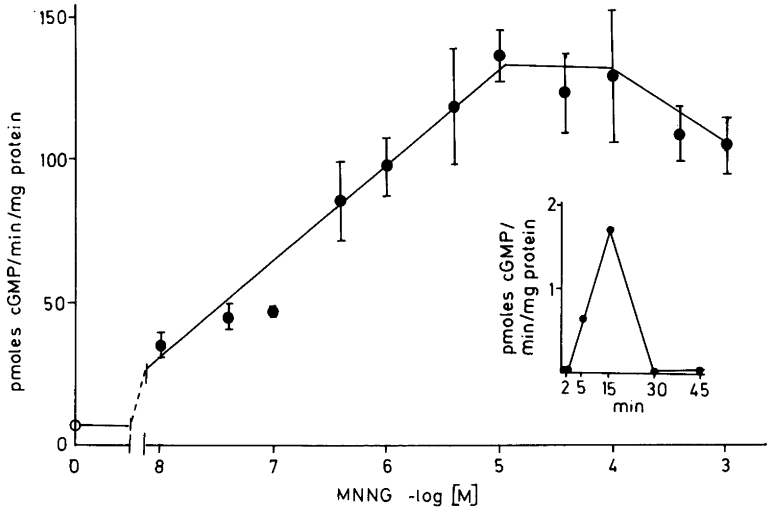

Fig. 1. Activation of hepatic nuclear guanylate cyclase by MNNG. The values represent the mean of 6 determinations, with the bars indicating S.E.M. Inset: accumulation of cGMP in nuclei isolated from liver slices.

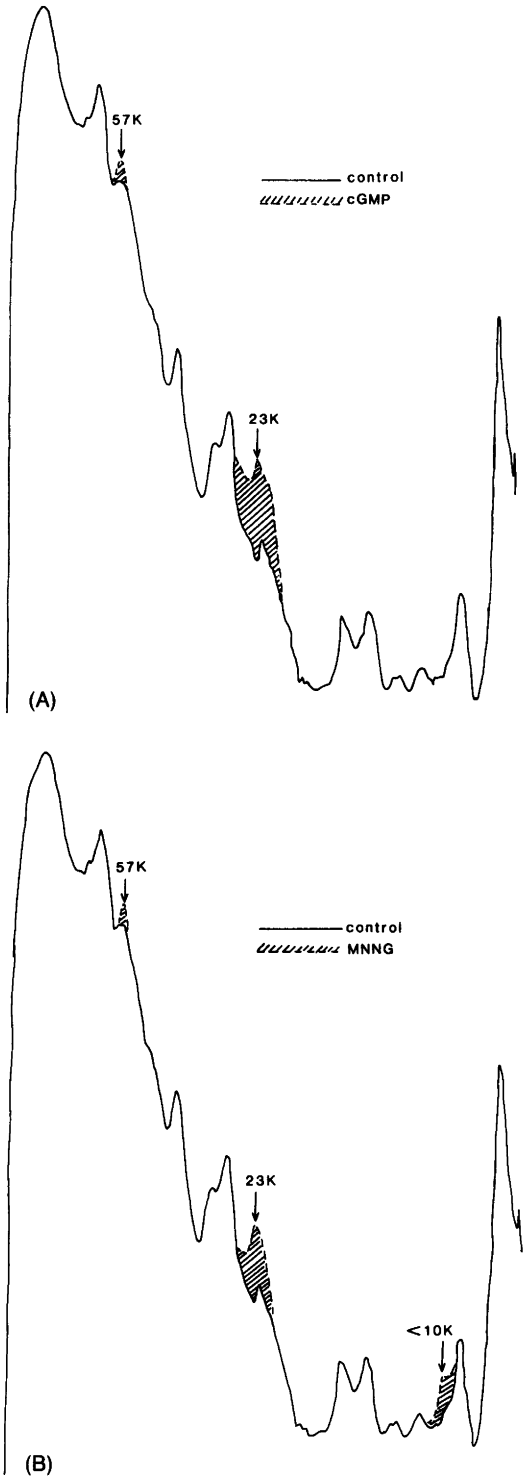

Fig. 2. Difference in the absorbance patterns of autoradiographs of SDS gels. Isolated nuclei were incubated with γ^{32}P-ATP in the absence and presence of cGMP (A) or MNNG (B). (A) Stimulation of $^{32}P\text{-}PO_4$ incorporation into two proteins (57 and 23 kD) in the presence of 5 μM cGMP. (B) Stimulation of $^{32}P\text{-}PO_4$ incorporation into two proteins (57 and 23 kD) in the presence of 100 μM MNNG.

in both nuclei and homogenates of regenerating liver (data not shown). cAMP (5 μM) did not stimulate phosphorylation of the 23,000 dalton protein or that of the 57,000 dalton protein in homogenates or in isolated nuclei.

Earlier studies have shown the presence of guanylate cyclase in the soluble, particulate and nuclear fraction from rat liver.[4] It was shown that nitrosoamines can activate the soluble and particulate guanylate cyclases.[4,5] The present study demonstrates that the nuclear guanylate cyclase can be activated to an even greater extent (22-fold) than the soluble and particulate guanylate (10–15-fold) cyclases found in homogenates. The elevated nuclear cGMP levels may reflect the 22-fold activation of the nuclear guanylate cyclase alone (Fig. 1), although a contribution by influx from the cytosolic cGMP pool during the preparation of nuclei cannot be excluded. The latter is a distinct possibility since cGMP can easily penetrate the nuclear envelope as shown by stimulation of protein phosphorylation in the nucleus by exogenously added cGMP (Fig. 2A).

The cGMP-dependent protein phosphorylation in isolated nuclei as shown here (Figs. 2A, B) demonstrates the presence of cGMP-dependent protein kinases within liver cell nuclei also. Whether the nuclear protein kinase activity reflects a cytosolic enzyme translocated into the nucleus similar to cAMP-dependent protein kinases in mammary tumours[5] cannot be concluded from this study. The identity of the two phosphoproteins in the nuclei which were phosphorylated in a cGMP-dependent manner remains to be established.

The finding that cGMP and MNNG will promote phosphorylation of proteins with identical mobility suggests that the effects of MNNG on nuclei are not confined only to methylation of DNA[3] but may also involve stimulation of cGMP-dependent protein phosphorylation. This may be involved in the MNNG-evoked activation

of DNA-polymerase.[16] It is suggested that this dual nuclear action should be taken into account in explaining the strong carcinogenity of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

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